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Although nearly half of human breast cancers at diagnosis are estrogen-responsive and respond to antiestrogen therapy at least for a time, more than half are estrogen receptor negative (ER-) and are predicted to be estrogen-nonresponsive tumors. However, some tumors which are ER- appear to respond to antiestrogen therapy in patients, which is beneficial because antihormonal therapy is less toxic than the alternative chemotherapy. The SCID mouse model has been recently used to study the hormonal dependence of growth of human breast cancer cells as tumors. Recent work has identified a potential model system where ER- breast cancer cells are estrogen-independent in cell culture, and yet intriguingly show estrogen-stimulated growth as tumors in SCID mice. This model system will extend understanding of the host contribution to tumor growth by isolating the host portion of estrogen-stimulated breast tumor growth. The potential to control tumor growth by modulating the host animal mechanism would be of particular value in the treatment of estrogen-nonresponsive tumors which recur after "breakthrough" of tamoxifen therapy, as well as tumors that are ER- and assumed to be estrogen-nonresponsive at diagnosis. Understanding how estrogens can lead to the increased growth of estrogen-nonresponsive breast tumors may lead to *new mechanisms* to better control the growth of estrogen-independent breast cancer.

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Introduction

Although nearly half of human breast cancers at diagnosis are estrogen-responsive and respond to antiestrogen therapy at least for a time, more than half are estrogen receptor negative (ER-) and are predicted to be estrogen-nonresponsive tumors (1). However, some tumors, which are ER-, appear to respond to antiestrogen therapy in patients, which is beneficial because antihormonal therapy is less toxic than the alternative chemotherapy. The athymic, *nude* mouse model (2) has been used to study the hormonal dependence of growth of human breast cancer cells as tumors. Recent work has identified a potential model system where ER- breast cancer cells are estrogen-independent in cell culture, and yet intriguingly show estrogen-stimulated growth as tumors in athymic, *nude* mice (3). This model system will extend understanding of the host contribution to tumor growth by isolating the host portion of estrogen-stimulated breast tumor growth. The potential to control tumor growth by modulating the host animal mechanism would be of particular value in the treatment of estrogen-nonresponsive tumors, which recur after "breakthrough" of tamoxifen therapy, as well as tumors that are ER- and assumed to be estrogen-nonresponsive at diagnosis. Understanding how estrogens can lead to the increased growth of estrogen-nonresponsive breast tumors may lead to *new strategies* to better control the growth of estrogen-independent breast cancer.

Our working hypothesis is that estrogen regulates breast tumor growth via both direct and indirect stimulatory paths. That is, both tumor cells and host cells, from the body in which the tumor resides, will respond to estrogen and contribute to tumor growth. Friedl and Jordan (3) have reported that estrogen will stimulate growth in estrogen receptor negative breast tumor cells (MDA-MB-231 subclone 10A) that have been implanted in athymic, *nude* mice. The authors proposed that estrogen stimulated normal mouse tissues to produce factors that promoted tumor growth through reduced tumor cell loss rather than through stimulation of tumor cell proliferation even though the cells within the tumor were not directly sensitive to estrogens. Our goal is to confirm this intriguing report by producing an athymic, *nude* mouse that lacks a functional ER and to determine the *in vivo* response of implanted breast cancer cell lines to estrogens.

Finally, we will establish and make available, as a service to the breast cancer research community, ER-minus mice in a *nude* mouse background to be used in model studies to help elucidate the direct and indirect *in vivo* effects of estrogens on breast cancer growth and on progression from estrogen dependence to independence.

Statement of Problem

MDA-MB-231 Subclone 10A cells are an estrogen receptor (ER)-negative, human breast cancer cell line that does not appear to respond to estradiol *in vitro*. Surprisingly, after implantation into athymic, *nude* mice (2), this ER-negative cell line exhibited estrogen-responsive growth as a tumor (3); treatment with estradiol over 9 weeks increased tumor growth (area) up to 10-fold. We propose to study ER- tumor growth in athymic, *nude* mice, in both wild type and ER "knockout" (ER-/-) mice, so as to be able to unambiguously determine the host animal contribution to estrogen-stimulated breast tumor growth. This system represents a unique and valuable model to study the host contribution to hormonal control of breast cancer but it is a high risk/ high benefit approach.

Why is Estradiol Responsiveness in Breast Cancer Tumors Clinically Important?

Breast cancer tumors that are estrogen responsive can be treated with relatively mild antihormonal therapy through the estrogen antagonist tamoxifen. However, most cancers become estrogen-independent, resulting in a resistance to treatment with tamoxifen (1). It would be extremely valuable clinically if we could make a breast tumor regain responsiveness to estrogen and eventually regain its dependence on estrogen. Alternatively, if we knew how a tumor cell lost its capacity to respond to estradiol then we might some day be able to prevent tumor cells from losing responsiveness to hormone treatment.

In this pilot study we will set up a model system that will allow us to study the mechanism by which a breast tumor cell line, lacking both its estrogen receptor and estrogen responsiveness *in vitro,* can exhibit estrogen responsiveness *in vivo*. Suggested mechanisms for this include estrogen responses in the host (not the tumor itself) which lead to increased angiogenesis of the tumor or reduced apoptosis of tumor cells. Our approach will use the ER knockout mouse as a model, which will separate host from tumor estrogen responses that lead to increased tumor growth. Understanding how estrogens can lead to the increased growth of estrogen-nonresponsive breast tumors may lead to new strategies to better control the growth of estrogen-independent breast cancer. Finally as a service to the scientific

community, we will establish and make available ER-minus mice in an athymic, *nude* mouse strain background so that both the indirect and direct *in vivo* effects of estrogens on other tumor cell lines can be examined.

Mechanism of ER Action

The estrogen receptor and its ligand, estradiol, have long been thought to be essential for survival (4,5), fertility, and female sexual differentiation and development. A potentially critical time of action for estrogen and its receptor is during blastocyst implantation and early embryogenesis. Support for this possibility was provided in a report by Hou and Gorski (6) in which reverse transcriptase-polymerase chain reaction (RT-PCR) was utilized to demonstrate expression of the ER gene in the mouse during the blastocyst stage of development. Using similar techniques, Wu et al. detected ER mRNA in both human (7) and mouse (8) oocytes, providing evidence for the potential role and importance of ER and estradiol even prior to fertilization. There is also no doubt that estrogen plays a central role in normal postnatal female physiology, as well as in female pathology where its importance in breast and uterine cancer, osteoporosis and cardiovascular disease is well known, although poorly understood.

ER, like all members of the extensively studied steroid receptor superfamily (9-11), is known to act through a now classic mechanism of ligand-dependent transcriptional regulation: 1) synthesis and secretion of estradiol in the ovary; 2) transport of the ligand usually associated with a serum binding protein in the blood stream; 3) passive diffusion through the cell membrane of the target tissues, uterus, breast, bone, etc.; 4) binding of the ligand to the receptor resulting in an allosteric receptor activation; 5) binding of a receptor dimer to a cognate DNA or hormone response element (HRE) usually located in the upstream promoter region of regulated genes; and finally 6) interacting with the transcriptional machinery and modulating transcription of specific genes.

Lack of Classical ER Responses in ER-Minus Mice

Consistent with the proposed crucial roles of estradiol and the ER protein, human ER gene mutations were unknown until recently (12). This is unlike the androgen receptor where many loss-of-function human mutations have been found (13-15). We have been able to generate mutant mice lacking responsiveness to estradiol (ER-minus mice) via gene targeting (16). Exon 2 of the ER gene was disrupted by insertion of a neomycin resistance gene. Both male and female ER- animals survive to adulthood with normal gross external phenotypes. No abnormalities have been detected in heterozygous animals. Homozygous females and males are infertile. Females have hypoplastic uteri and "hyperemic" ovaries with no detectable corpora lutea.

Classic biological assays for estradiol are also unresponsive: In wild type and heterozygous females, three day estradiol treatment with 40 ug/kg dose stimulates a 3 to 4 fold increase in uterine wet weight and alters vaginal cornification, but the uteri and vagina of the ER-disrupted animals do not respond. Prenatal male and female reproductive tract development can therefore occur in the absence of estradiol receptor-mediated responsiveness.

ERα-Minus Mice PCR analysis, RNase protection, Western Northern and Southern Nucleic Acid Analysis to Check for Functional Estrogen Receptor

Western blot protein analysis does not detect any classic ER protein in ER-minus mice. Northern blot analysis of poly A-mRNA isolated from ER minus uteri and probed with either a radioactive ER cDNA probe or neomycin gene probe detected the same size band. Southern blot analysis with ER and neomycin probes shows the expected 2 kb increase in size of the band from the ER gene because of the insertion of the 2 kb neomycin resistance gene. The presence of a single band with neomycin and ER probes indicates that there is only one insertional disruption integration site and that it is in the ER gene.

RNase protection studies with ligand-binding domain probes reveal that the ER-minus mice contain less than 20% of the level of mRNA in this region when compared to wild type ER mRNA. No mRNA containing the full length wild type sequence is detected by PCR although small incomplete mRNA fragments that use cryptic and native donor/acceptor splice sites are detected indicative of neomycin gene disruption of the ER gene transcript. This indicates as expected that some disrupted ER mRNA is being made. It is inactivated as expected by the inserted neomycin gene, because no classic ER protein functional responses are detected.

ER-Minus Mice Biological Responses

We have been concerned that ER protein might somehow be present by "leakage" in the ER-minus mice. Several experiments on the ER-minus mice phenotype in classic estrogen response assays have been performed since the publication of the original ER-minus mouse paper (16,18). We have been unable to detect any classic estradiol responses utilizing several classic biological estrogen assays and transcription-mediated assays in these ER-minus animals.

The biological assay results showing a lack of estradiol response are listed below:

1. Estradiol, as found previously, has reproducibly been unable to increase uterine wet weight and vaginal cornification.
2. Previously it has been found that uteri would respond to epidermal growth factor (EGF) mediated by ER protein but independent of estradiol. There was no response to EGF in these mice, again indicating the absence of functional ER protein.
3. Tamoxifen and diethylstilbestrol (DES) have estrogenic activity in the uterine wet weight gain assay in wild type animals. No effect on uterine wet weight was seen after administration to the ER-minus animals. It has been speculated that there are alternative steroid receptor super family orphan receptors, in addition to ER, for tamoxifen and DES. If these additional receptors exist, they are not able to function to increase uterine wet weight.
4. In other estradiol biological response assays in ER-minus mice, estradiol is also unable to show any response. No increased response was seen after estradiol administration in: a) in ^{3}H-thymidine incorporation in in vitro uterine uptake assays, b) in PR mRNA levels, c) in glucose 6-phosphate dehydrogenase mRNA levels, and d) in lactoferrin mRNA levels. These are very sensitive transcriptionally mediated, classic estradiol response assays. The lactoferrin mRNA assay, for example, shows over a 350-fold stimulation in wild type animals after exposure to estradiol.
5. Recent results have shown by in situ hybridization assays that 12 hour exposure to 4-hydroxy catechol estrogen but not estradiol can induce lactoferrin mRNA in ovariectomized ER-minus mice uteri. This result provides support for the existence of at least one additional "estrogen" response protein capable of responding to an estrogen "metabolite".

Body

Hypothesis/Purpose:

We propose that cells of host tissue respond to estrogen by producing a "factor" capable of stimulating breast tumor cell growth. Confirmation that host responsiveness to estradiol is required for tumor growth will be sought by implanting the ER-minus breast tumor cell line, MDA-MB-231 (subclone 10A), in *nude (later changed to SCID for technical reasons described below)* ER-minus mice lacking estrogen responsiveness. If a host response to estradiol is required for tumor growth then the tumor will not respond in ER-minus mice.

The lack of ER-negative tumor response to estradiol in the ER-minus mice provides support for these two possible mechanisms:

1. The breast tumor cells are responding indirectly to estrogen, perhaps through a factor induced in another tissue in the mouse that is altering tumor cell proliferation and/or apoptosis.
2. Estradiol increases the rate of angiogenesis and nutrient supply thus allowing faster tumor growth.

If, as we expect, the ER-negative tumor cells are non-responsive to estradiol *in vivo*, then the case for direct stimulation by the following mechanisms is weakened:

1. The tumor cells regain functional estrogen receptor protein *in vivo*.
2. One of the other known non-ER, estrogen-binding proteins, perhaps capable of mediating a growth response, is induced *in vivo*.

Original Technical Objectives:

Technical Objective #1.

We will establish a colony of athymic, *nude* mice and ER+/- mouse heterozygotes to produce 5-15 animals per week of female athymic, *nude* ER-/- mice and control female athymic, ER+/+ mice sufficient for the technical objectives.

Technical Objective #2.

A. While we are establishing the colony of athymic, *nude*, ER-minus mice, we will use commercially available athymic, *nude* mice (ER-positive) to confirm that the estrogen receptor-negative MDA-MB-231, subclone 10A, breast cancer cell line will not respond to estrogen in *in vitro* culture but will respond *in vivo* in the athymic, *nude* mouse. We will use estrogen-dependent MCF-7 cells as positive controls for estrogen responsiveness and parental MDA-MB-231 cells as controls for estrogen-independence. (See Table 1) Both of these human breast cancer cell lines have been used extensively in the athymic mouse model. We expect that MCF-7 cells will grow as tumors that require estrogen in the athymic mice, and that parental MDA-MB-231 cells will grow as tumors independent of estrogens. Furthermore, we expect that estrogens will increase the growth of the subclone 10A cells only in the ER-positive mice, confirming the results of Friedl and Jordan (3). We will also test the specificity of this response with estrone, estriol, tamoxifen, the antiestrogen, ICI 164,384 and catechol estrogen to determine if the response is mediated by ER protein of the host animal.

B. We will confirm that the subclone 10A tumors lack estradiol-binding activity at the end of the experiments, as well as ER protein and mRNA both *in vitro* and *in vivo*. We will assay for ER protein by estradiol binding activity and Western blot analysis and assay for mRNA by RNase protection and quantitative polymerase chain reaction (PCR) analysis. We expect that the tumors will lack ER in any form both *in vitro* and *in vivo*.

Another possible result, almost as intriguing is that the tumors possess ER protein or mRNA either *in vitro* or *in vivo*, but it is only functional *in vivo* or it is only induced *in vivo*. (We consider this unlikely but if it happens, see Future Directions below.

Technical Objective #3.

We will test the hypothesis that the ER-negative tumor cell line, MDA-MB-231 subclone 10A, is being indirectly influenced by factors induced by estradiol acting through ER protein in tissues outside the tumor. We will implant the ER-minus tumor cell line into a female *nude*, ER-minus mouse. (See Table 1) We have already made an ER-minus mouse lacking a functional ER protein by disrupting the ER gene by homologous recombination. We will cross this ER-minus mouse with an athymic, *nude* mouse on a C57BL background. The resulting athymic, ER-minus mouse will allow the implantation of exogenous tumor cells into a mouse lacking responsiveness to estradiol. We can then determine whether the growth response of the tumor cells to estradiol is mediated directly or indirectly by estradiol. If our hypothesis is correct, the *in vivo* tumor cell line should not respond to estradiol in an environment lacking a functional ER. Further characterization of the putative growth factors could then be undertaken by methods briefly outlined in Future Directions below. A positive response to estradiol would suggest that a search should be begun, both within the tumor cell line and within the ER-minus mouse, for a novel non-ER, estrogen response protein.

We will use estrogen-dependent MCF-7 cells as positive controls for estrogen responsiveness and parental MDA-MB-231 cells as controls for estrogen-independence. Both of these human breast cancer cell lines have been used extensively in the athymic, *nude* mouse model. We expect that MCF-7 cells will grow as tumors that require estrogen in both ER-positive and ER-minus athymic mice, and that parental MDA-MB-231 cells will grow as tumors independent of estrogens in both mice. However, we expect that estrogens will increase the growth of the subclone 10A cells only in the ER-positive mice.

Technical Objective #4.

From the estrogen-responsive MCF-7 human breast cancer cell line, we have derived several unique clonal sublines, which are estrogen-independent for proliferation in cell culture (17). We will screen these cell lines for estrogen-dependence of tumor growth in the athymic, *nude* ER-minus mouse model that we have described above. (See Table 1) The goal is to identify other cells lines, which may contribute to the study of the estrogen-stimulated host response and test the generality of ER-minus tumors responding to estrogens.

ER-Minus, Estrogen-Nonresponsive Cell Lines Derived from the Estrogen-Dependent MCF-7 Human Breast Cancer Cells

The MCF-7 human breast cancer cell line is widely used as a model of hormone-responsive breast cancer. These cells contain ER, progesterone receptors (PgR), and show estrogen-dependent cell proliferation. By culture under hormone-free conditions, Wade Welshons' laboratory has isolated two intermediate cell lines in progression from the ER+, PgR+, estrogen-responsive phenotype of the parental MCF-7 cells, to the ER-, PgR- and estrogen-nonresponsive *in vitro* phenotype (17). This progressive series of cell lines follows the sequence: loss of PgR, loss of estrogen-responsive proliferation with reduced ER, and finally complete loss of ER. Several subcloned cell lines of each phenotype have been isolated. All ER-minus cell lines fail to express ER mRNA by the RNase protection assay, but all cell lines have an apparently intact ER gene by genomic Southern analysis (17). In Technical Objective #4, we propose to determine if any estrogen-nonresponsive clones in this series will, like subclone 10A proposed for extensive study above, exhibit estrogen-stimulated growth as tumors in athymic mice. This will test the generality of the phenomena that estradiol can exert positive effects on tumor growth through host mediated action.

Table 1

Expected Growth Responses to Estrogen Treatment of Breast Cancer Cells (with and without ER) Implanted in ER wt & ER - athymic, *nude* Mice

	A	***B***	***C***	***D***
	MCF-7 (ER-positive)	MDA (ER-minus)	Subclone 10A (ER-minus)	Our MCF-7 clones (ER-minus)
Nude ER wt/wt mice				
no E2	–	+++	+	+ (?)
with E2	+++	+++	+++	???
Nude ER -/ - mice				
no E2	–(?)	+++(?)	+(?)	+ (?)
with E2	++(?)	+++(?)	+(?)	+ (?)

Brief description of breast cancer cells above that are to be implanted into ovariectomized mice for tumor growth experiments:

A. MCF-7: ER-positive, estrogen-responsive for proliferation in culture and as tumors

B. MDA-MB-231 parental: ER-minus, estrogen-nonresponsive for proliferation in culture and as tumors

C. Subclone 10A: Derived from MDA-MB-231; ER-minus, estrogen-nonresponsive for proliferation in culture, but estrogen-responsive for growth as tumors

D. MCF-7 clones (several): ER-minus, estrogen-nonresponsive for proliferation in culture, estrogen-responsiveness unknown as tumors (Isolated in Wade Welshons' laboratory by Ed Curran.)

Original Methods: (Some detailed updated methods are found in published papers from this grant. One is attached here (17e); the other was attached to a previous report (17d).)

Establishment of a Breeding Colony of Athymic, *Nude* ER-Minus Mice (now changed to SCID/ER-minus because of breeding problems with nude mice)

The crossing of the ER-minus mouse into the athymic, *nude* background will require four generations before we can routinely produce ER-minus, *nude* female mice capable of accepting breast tumor xenografts.

Because the *nude* mice are immune compromised, all breeding with these mice will be done under microisolater conditions with everything coming in contact with the animals being first autoclaved. The animals will be taken out of their microisolaters only under a laminar flow hood to help maintain sterility. Genotyping of the mice pups will be by appearance for the *nude* genotype and by multiplex PCR for the ER-minus genotype. Multiplex PCR genotyping is

routinely done for our ER-minus colony on about 200 DNA samples per week, isolated from tail snips of five week old mice. This same procedure and scale will be followed for the *nude*, ER-minus mouse colony.

In the first generation two *nude* C57BL/6J males, obtained from Jackson Laboratories, will be crossed with new pairs of heterozygous ER-minus female mice every two weeks. (See Figure 1) (The C57BL/6J strain background was chosen because it is the strain into which our ER-minus colony is being backcrossed to remove the 129 background of the original male founder.) Female *nude* mice are poor mothers so they are not useful in efficiently generating offspring. These multiple initial matings will efficiently generate double heterozygous ER-minus/*nude* females at a 1 in 8 frequency. (This frequency is the best possible whenever dealing with ER-minus crosses because heterozygotes must be used yielding only 1 in 4 ER-minus animals per mating, of which only half are the desired sex.) In the second generation the double heterozygote females will be crossed with the males of the same genotype as their father. This backcross will generate both female offspring with the same genotype as the mother (frequency of 1/8) and male *nude,* heterozygote ER-minus offspring (frequency of 1/8). These two genotypes will be crossed in the third generation to produce female *nude*, ER-minus mice at a frequency of 1 in 16. Their siblings will be used to set up additional breeding pairs to produce additional female *nude*, ER-minus mice. It is estimated that 40 litters of 8 pups each will be necessary to produce the 20 female ER-minus, *nude* mice necessary for a typical experiment.

Cell Culture and ER Analysis

The breast cancer cell lines will be maintained in Minimum Essential Medium (MEM) containing nonessential amino acids, HEPES 10 mM, insulin 6 ng/ml, penicillin 100 μg/ml, streptomycin 100 units/ml, and 5% bovine calf serum (maintenance medium). For assay of estrogen stimulated proliferation, cells are seeded in estrogen-free medium (maintenance medium prepared with phenol red-free MEM and 5% of charcoal-stripped calf serum) in 24-well plates, and after 3 days incubation estrogen-free, are treated for 4 days with estrogen-free medium that contains estrogens or test compounds, with proliferation determined by measurement of DNA at the end of the experiment (19). Estrogen receptors and progesterone receptors will be determined by whole cell uptake of [^{3}H]estradiol or [^{3}H]R5020, respectively (19); whole cell uptake is very sensitive and accurate for low levels of receptors in cells *in vitro*. The presence of estrogen receptor protein and mRNA are routinely determined in our lab by SDS gel separation/Western blotting for the protein, and by solution hybridization/RNase protection for the message. Sensitive quantitation of possible low level ER will be performed by quantitative PCR (20).

Tumor Implantation and Measurement

Cells for implant to form tumors will be cultured to confluence in 15 cm^2 culture dishes and scraped in 1 mM EDTA in calcium-magnesium-free Hanks' balanced salt solution (CMFH), washed with MEM and resuspended to approximately 30 x 10^6 cells/ml. Three million cells in suspension (0.1 ml) will be injected into the axillary mammary fat pad of ovariectomized athymic mice 5-7 weeks of age; over half of the implants are expected to develop into tumors. Tumor area will be measured weekly from the greatest (length) and narrowest (width) dimensions of the tumor measured through the skin, and area will be calculated as length/2 x width/2 x pi. Ten to twelve animals will be treated with estrogen by implanting commercial pellets of estradiol (Innovative Research, Toledo, OH), and of other estrogens and antiestrogens by silastic tubing as described (21).

Key Research Accomplishments/Progress

Technical Objective #1

Establishment of a Breeding Colony of Athymic, *Nude* ER-Minus Mice

Original Plan:

Colony will be up to size by end of first year of funding and be maintained through end of grant period.

The crossing of the ER-minus mouse into the athymic, *nude* background will require four generations before we can routinely produce ER-minus, *nude* female mice capable of accepting breast tumor xenografts Because the *nude* mice are immune compromised, all breeding with these mice will be done under microisolater conditions with everything coming in contact with the animals being first autoclaved. The animals will be taken out of their microisolaters only under a laminar flow hood to help maintain sterility. Genotyping of the mice pups will be by appearance for the *nude* genotype and by multiplex PCR for the ER-minus genotype. Multiplex PCR genotyping is routinely done for our ER-minus colony on about 200 DNA samples per week, isolated from tail snips of five week old mice. This same procedure and scale will be followed for the *nude*, ER-minus mouse colony.

In the first generation two *nude* C57BL/6J males, obtained from Jackson Laboratories, will be crossed with new pairs of heterozygous ER-minus female mice every two weeks. (See Figure 1) (The C57BL/6J strain background was chosen because it is the strain into which our ER-minus colony is being backcrossed to remove the 129 background of the original male founder.) Female *nude* mice are poor mothers so they are not useful in efficiently generating offspring. These multiple initial matings will efficiently generate double heterozygous ER-minus/*nude* females at a 1 in 8 frequency. (This frequency is the best possible whenever dealing with ER-minus crosses because heterozygotes must be used yielding only 1 in 4 ER-minus animals per mating, of which only half are the desired sex.) In the second generation the double heterozygote females will be crossed with the males of the same genotype as their father. This backcross will generate both female offspring with the same genotype as the mother (frequency of 1/8) and male *nude,* heterozygote ER-minus offspring (frequency of 1/8). These two genotypes will be crossed in the third generation to produce female *nude*, ER-minus mice at a frequency of 1 in 16. Their siblings will be used to set up additional breeding pairs to produce additional female *nude*, ER-minus mice. It is estimated that 40 litters of 8 pups each will be necessary to produce the 20 female ER-minus, *nude* mice necessary for a typical experiment.

Results 1997:

Progress so far has been frustrating. The success of this objective is dependent on the successful breeding of sufficient numbers of female *nude*/ERαKO mice to be immune compromised recipients of xenotransplants of human breast cell lines to determine their responses to estrogens. This objective is essential for the project to succeed. Unfortunately because of two main findings we have had to alter our approach to this objective. Our first finding was that litter size has been half that expected. Our second finding was that the ratio of male to female pups was 2:1. While these may be from a basic science point of view fascinating, they have hindered the accomplishment of this objective. These two findings have taken our expected ratio of 1 in 16 pups (to be the correct sex and genotype) down to about 1 in 32 in twice the number of litters. As a result we have only been able to breed less than 10 mice of the correct genotype and sex and these have been of unmatched ages, which have not allowed us to run controlled experiments.

Fortunately, we have developed and begun to implement a new approach, which should overcome our problems with using *nude* mice. This is to substitute SCID (severe combined immuno-deficient) mice in a C57BL/6J background obtained from Jackson Laboratories. These animals have recently begun to be used in other laboratories to replace *nudes* in breast cancer xenotranplantation studies. They will be important because unlike the *nude* mice, they are more prolific breeders and more importantly the homozygous females are fertile. This should allow us to approach a 1 in 8 frequency.

Results 1998:

The good news is that we have finally been able to put heterozygous ERaKO mice on a homozygous SCID background. We have within the last month started making the crosses that should give us KO mice and WT controls on a SCID background so that we can do the transplantation experiments planned in #2 below. The bad news is that what should have taken 9 months and 3 generations instead took 15 months because of problems in distinguishing the homozygous SCID mice from the heterozygous SCID mice. Additionally, the litter size is down to about 6 per litter but at least we will be able to get mice that we can start to work on!

Technical Objective #2

Results 1997:

The discovery of a second estrogen receptor, ER-beta (17b), as well as our own work that a third estrogen receptor (ER-gamma?) may exist (17c), has confounded this objective. We have developed PCR primers to check for both ER-α, and ER-beta mRNAs to clarify the true of ER status of published "ER-negative" human breast cell lines. These studies are ongoing.

Results 1998:

See new Figure 2. It is clear that ER-beta mRNA is present at low concentrations in most or all supposedly "ER-negative" breast cancer cell lines. These lines display no detectable estrogen binding so the ER-beta protein is either at too low a concentration to be of importance or it is an alternate spliced form that cannot bind estrogens. We have preliminary evidence that it is the later in that only a deleted form of the ER-beta mRNA can be seen by RT-PCR (data not shown.)

Technical Objective #3

Results 1997:

No ERaKO/*nude* mice so no results yet. See Objective #1 results above.

Results 1998:

We have finally begun to implant cell lines into a few of our first SCID ERaKO mice. No results yet. See #1 above.

Additional Detailed Methods 1999:

Using mice produced from technical objective #1, we have attempted to grow tumors in wild and ERaKO mice from implants of MCF-7 cell suspensions using procedures observed by postdoctoral fellow, Ed Curran, in the laboratory of Dr. Osborne at the University of Texas Medical Center - San Antonio, Texas. Six to eight week old female mice were ovariectomized using sterile techniques. The ovariectomized mice were allowed to recover from surgery for at least one week, at which time 3-week release pellets containing 0.125 mg estradiol/pellet (Innovative Research, Sarasota, Fl.) were implanted subcutaneously in each mouse. The MCF-7 cells used in these studies were originally obtained from the laboratory of Dr. Craig Jordan (3), Northwestern University. The cells were grown in T150 tissue culture flasks in MEM medium supplemented with 5% fetal bovine serum. Two to three days after implantation of the estradiol-releasing pellets, the cells to be implanted were scraped off the flasks with rubber policemen, pelleted and resuspended at 15 x 106 cells/200 ul. 200 ul of cell suspension was injected near the right axillary mammary fat pad in each mouse. The mice were observed daily for at least 3 weeks after injection for evidence of a palpable tumor mass. In some experiments, at the end of three weeks an additional 3-week release pellet containing 0.125 mg estradiol was implanted into mice that had already been injected with MCF-7 cells. These mice were observed an additional 3 weeks for evidence of tumor masses. C57Bl6J and BALBC/J mice were also obtained from Jackson Laboratories, Bar Harbor, Maine as control animals.

Results 1999:

In three separate trials involving at least 4 wild type scid/scid and 4 ERaKO scid/scid mice per trial we were unable to observe palpable tumors in either the wild type or ERaKO mice 3 weeks after injection of the MCF-7 cells. Some of these mice were also necropsied by Drs. Besch-Williford and Franklin (both DVMs) and they were unable to find tumors in these mice. We also implanted MCF-7 cells in wild type nu/nu and ERaKO nu/nu mice and again were unable to observe tumor growth in either the wild type or ERaKO nude mice. Our inability to grow tumors in the C57Bl6J scid/scid and C57Bl6J nu/nu mice that we produced led us to attempt to grow MCF-7 tumors in mice obtained from Jackson Labs. Because many labs that use xenograft model of tumor growth use the BALBC strain of mouse we also decided to compare tumor growth in BALBC scid/scid and C57Bl6J scid/scid strains. In this experiment we were able to obtain palpable tumors in 2/2 mice of the BALBC strain but no tumors were observed in 2 C57Bl6/J strain mice. This indicates that strain differences between BALBC and C57Bl6J may in part account for our inability to produce MCF-7 tumors in our mice, as they are all C57Bl6J.

Another potential explanation for our inability to grow tumors in our mice is the fact that our SCID mice have contracted several opportunistic infections during the initial breeding scheme at which time we were crossing the ERaKO gene into the SCID and NUDE backgrounds. The infections include Pasteurella pneumotropica, Pneumocystis carinii, mouse cytomegalovirus (MCMV) and Helicobacter hepaticus. These organisms are present at background levels in immunocompetent animals such as our normal ERaKO colony. We were unaware that these opportunistic pathogens were being transmitted during our breeding scheme. Dr. Shafie at Innovative Research believes that tumors cannot be grown in

an unhealthy mouse because the immune system is activated. [See additional thoughts (under Reportable Outcomes below) and future work ideas that we are working on since the funding for this grant has ended.]

Technical Objective #4

Results 1997:

With research progressing so slowly on the first three objectives we have turned our attention to this objective. We have analyzed the MDA cell line and the MCF-7 cell lines developed by Curran and Welshons (See Figure 1) and have 2 significant results to report from analyzing these lines

One of the accepted dogmas for the inactivation of ER in breast tumors, so that it is no longer responsive to estrogens, has been that when DNA methylation occurs at the NotI site of the ER gene, its transcription is turned off. Thus methylation and perhaps demethylation might allow ER-negative tumors to be reactivate their ER genes and subsequent responsiveness. We have shown that in these MCF cell lines there is not a correlation of methylation and ER expression (Cancer Research *submitted*). In the process of looking for correlation of methylation at other DNA sites of expression of the ER gene we have developed a technique that allows for the entire genome to be scanned to look for differences in DNA methylation (Ref 17d Huang *et al.*; and 17e Laux *et al.* See Appendix for this new Breast Cancer Research and Treatment paper). In particular this exciting technique has allowed the discovery of a novel tumor suppressor gene (WT1) that is apparently inactivated in breast cancer. The role of estrogen in regulating this tumor suppressor is at present unclear.

If ER negative cell lines are becoming responsive to estrogens then perhaps it is not ER-alpha that is being reactivated but rather ER-beta or another ER. Our second significant result is the utilization of an RT-PCR assay to analyze for ER-beta mRNA in these MDA and MCF-7 cell lines (Figure 1). *The data presented in OLD Figure 2 (NOT SHOWN HERE because it is incorrect) (lanes 1, 4, 7, 10, 13, 16, 19, 22) clearly shows that ER-beta mRNA is not present in any of the breast cancer cell lines but is present in the positive control human testes RNA (Figure 2 lane 25).* We now believe this to be incorrect! Small bands in these lanes are non-specific primer dimers. Other controls show that RNA is present in all samples because actin and RLP7 mRNAs are present in all samples.

Results 1998:

See # 2 above and NEW Figure 2 and legend. The old Figure 2 above was incorrect and has been eliminated.

Results 1999:

No additional progress because of Task #3 results (see above).

1998 Time Line (Because of results from Technical Objective #3 or Task #3 above this time line has been disrupted.)

Technical Objective #1

Establishment of a Breeding Colony of *SCID* ER-alpha Minus Mice

This is essentially done. We are just beginning to obtain sufficient pups to screen and age for studies below.

Technical Objective #2/#3, and #4 have been combined and rearranged in order to deal with problems encountered in setting up the SCID/ERaKO transgenics.

We plan to complete all of the objectives by November 1999 (Did not happen. See Tech. Objective #3 above), IF no more unforeseen problems occur (They did occur.). These mice have been a *bear* to obtain. However with them a lot of additional experiments outside the scope of this now un-funded proposal can now be done to facilitate our understanding of estrogens role in breast cancer. We hope to put in for additional funding in the next go around.

First Experiment: Implantation of MCF-7 cells.

4 Groups-10 animals per group- 4 injection sites per animal-2-5x10^6 MCF-7 cells per injection site

Ovariectomized WT SCID mice-not treated
Ovariectomized WT SCID mice-treated with E2 silastic implants
Ovariectomized ERαKO SCID mice-not treated
Ovariectomized ERαKO SCID mice-treated with E2 silastic implants

First animals are 3-4 weeks old; ovexing of first animals will begin the second week of December with tumor implantation beginning 1 week later. Tumors will be allowed to grow for approximately 2-3 months, during which time tumor volume measurements will be made when palpable tumors appear. At this time the animals will be sacrificed and tumors and adjacent mammary tissue will be collected and analyzed for ER expression (ligand binding), tumor morphology including vascularization will be evaluated by standard histological and morphometric techniques, and RNA will be isolated for future analysis of such things as expression of growth factors and angiogenesis factors. To accumulate 10 animals in each group will probably take an additional 4 months from the completion of the first animals.

Second Experiment: Implantation of MDA-MB-231, subclone 10A, cells.

4 Groups-10 animals per group- 4 injection sites per animal-2-5x10^6 MDA-MB-231, subclone 10A cells per injection site

Ovariectomized WT SCID mice-not treated
Ovariectomized WT SCID mice-treated with E2 silastic implants
Ovariectomized ERαKO SCID mice-not treated
Ovariectomized ERαKO SCID mice-treated with E2 silastic implants

Studies will begin, as animals become available towards the end of the first experiment, which should be mid February to mid April. Tumors will be allowed to grow for approximately 2-3 months, during which time tumor volume measurements will be made when palpable tumors appear. At this time the animals will be sacrificed and tumors and adjacent mammary tissue will be collected and analyzed for ER expression (ligand binding), tumor morphology including vascularization will be evaluated by standard histological and morphometric techniques, and RNA will be isolated for future analysis of such things as expression of growth factors and angiogenesis factors. To accumulate 10 animals in each group will probably take an additional 3-5 months from the completion of the first animals.

Third Experiment: Implantation of MCF-7 and other ER-negative cell lines.

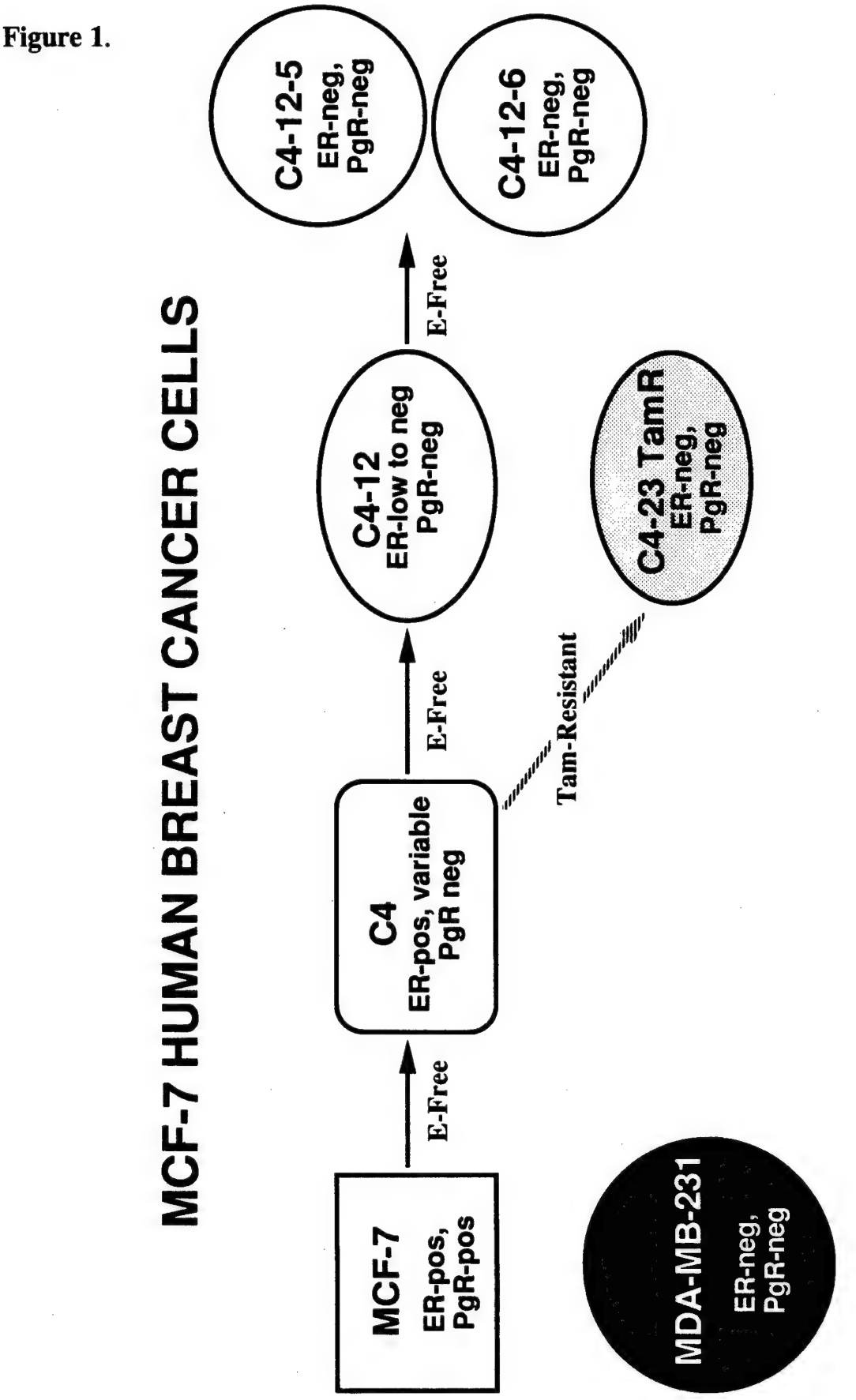
10 Groups-5 animals per group-4 injection sites per animal

1. WT SCID mice- 2-5x10^6 MCF-7 cells per injection site
2. WT SCID mice- 2-5x10^6 MDA-MB-231, subclone 10A (ER-negative) per injection site
3. WT SCID mice- 2-5x10^6 MDA-MB-231 (ER-negative) cells per injection site
4. WT SCID mice- 2-5x10^6 C4-12-5 (ER-negative) cells per injection site
5. WT SCID mice- 2-5x10^6 C4-12-6 (ER-negative) cells per injection site
6. ERαKO SCID mice- 2-5x10^6 MCF-7 cells per injection site
7. ERαKO SCID mice- 2-5x10^6 MDA-MB-231, subclone 10A (ER-negative) per injection site
8. ERαKO SCID mice- 2-5x10^6 MDA-MB-231 cells (ER-negative) per injection site
9. ERαKO SCID mice- 2-5x10^6 C4-12-5 cells (ER-negative) per injection site

10. ERαKO SCID mice- 2-5x10^6 C4-12-6 cells (ER-negative) per injection site

Studies will begin, as animals become available towards the end of the second experiment, which should be mid June to mid August. Tumors will be allowed to grow for approximately 2-3 months, during which time tumor volume measurements will be made when palpable tumors appear. At this time the animals will be sacrificed and tumors and adjacent mammary tissue will be collected and analyzed for ER expression (ligand binding), tumor morphology. Standard histological and morphometric techniques will evaluate vascularization, and RNA will be isolated for future analysis of such things as expression of growth factors and angiogenesis factors. To accumulate 5 animals in each group will probably take an additional 4 months from the completion of the second animals.

Figure 1.


MCF-7 HUMAN BREAST CANCER CELLS
MCF-7
ER-pos,
PgR-pos
E-Free
C4
ER-pos, variable
PgR neg
E-Free
C4-12
ER-low to neg
PgR-neg
E-Free
C4-12-5
ER-neg,
PgR-neg
C4-12-6
ER-neg,
PgR-neg
Tam-Resistant
C4-23 TamR
ER-neg,
PgR-neg
MDA-MB-231
ER-neg,
PgR-neg

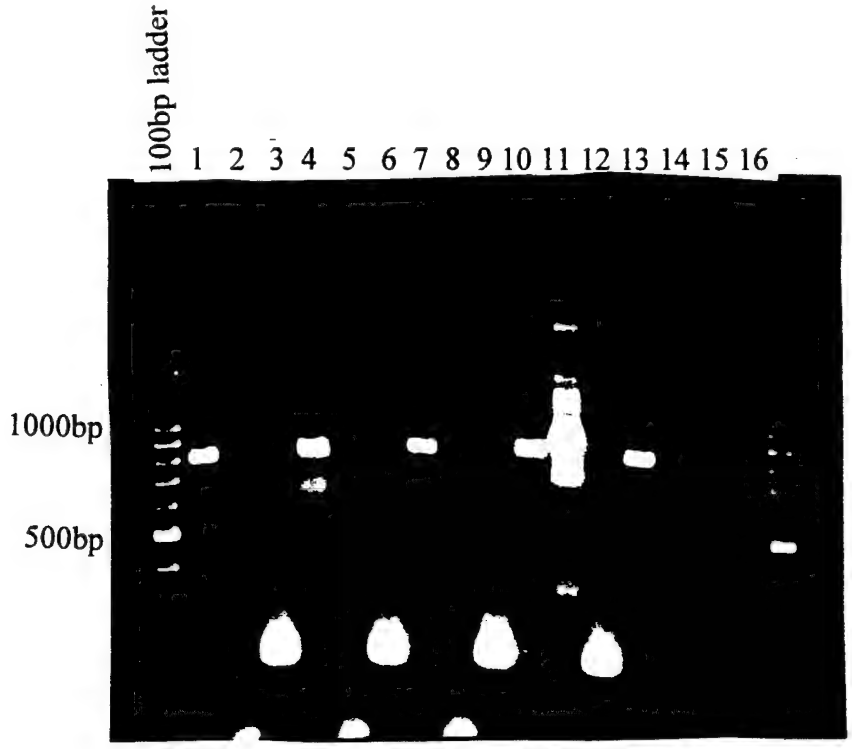


Figure 2. RT-PCR of RNA from the following cell lines amplified with primers for either ERα, ERβ, or the internal standard GAPDH

These findings demonstrate that the parent breast cancer cell line (MCF-7) made large amounts of ER-alpha mRNA and low amounts of ER-beta mRNA. The derived cell lines (C-4-12-5 and C-4-12-6) (as well as the MDA cell line) contained little or no ER-alpha mRNA and the same amount of ER-beta mRNA as the parent MCF-7. The MDA line is similar to the one used by Craig Jordan that was "ER negative" but responded to estrogens *in vivo*. In data not shown the ligand-binding domain of the ER-beta is alternatively spliced and is incapable of binding estrogens.

C-4-12-5 cell line: Lanes 1-3
Lane 1 ERβ
Lane 2 ERα
Lane 3 GAPDH

C-4-12-6 cell line: Lanes 4-6
Lane 4 ERβ
Lane 5 ERα
Lane 6 GAPDH

MDA cell line: Lanes 7-9
Lane 7 ERβ
Lane 8 ERα
Lane 9 GAPDH

MCF-7 parent cell line: Lanes 10-12
Lane 10 ERβ
Lane 11 ERα
Lane 12 GAPDH

Human Bladder Pos. Control Lanes 13-14
Lane 13 ERβ
Lane 14 ERα

H_2O Negative Control: Lanes 15-16
Lane 15 ERβ
Lane 16 ERα

Reportable Outcomes and Future Studies:

1) A Cancer Research paper was attached last year: Huang *et al.* 1997. (Reference 17d).

2) A new Breast Cancer Research and Treatment paper is attached. Laux *et al.* 1999 (Reference 17e) See Appendix where it is attached.

3) Abstract for 2nd ERA of Hope meeting in October 2000 (See Appendix)

4) With the data generated from these studies additional outside funding through NIH (1P01 ES 10535-01 pending) has been obtained to study estrogen and phytoestrogen effects on the immune system, which may likely have a direct effect on breast cancer response to estrogens.

Future Directions Planned for 2000:

We believe that this is still an important study, despite all of the technical difficulties, and we are taking or plan to take the following steps to make this project work:

1. We are re-deriving the ERαKO SCID colony by embryo transfer to eliminate opportunistic infectious agents in the colony. This is well underway and we have pups from embryo transfer we can breed and test for the presence of these opportunistic infectious agents. "Cleaning" the mice alone may allow tumors to take in the ERαKO SCID/ C57Bl6J mouse
2. We are searching for more data on growth of breast cancer cell lines in a C57Bl6 background - by far the most common background in mice used in these studies is BALBC. C57Bl6J mice are known to have a more aggressive immune system, including increased NK cell activity. This may in part explain our inability to grow tumors in the C57Bl6J background.
3. If indeed the C57Bl6J background is more difficult to grow tumors in then we will try to remedy this problem. Potential solutions include depressing NK cells with an injection of a NK cell specific antibody one day prior to tumor injections. This should suppress NK cell activity and may allow enough time for the tumor to take hold before NK cell activity returns. Also injection of Matrigel with the tumor cells may allow tumor to take in the more hostile environment the C57Bl6J mouse.
4. The production of the ERαKO/SCID/C57BL6J mice has allowed us to examine the innate immune system in the absence of the ERα and the acquired immune system (T and B lymphocytes). The effect of estrogens on the immune system is likely have an important effect on mammary tumor growth.

Additional Possible Long Term Future Directions Resulting from this Proposal:

Angiogenesis

Estrogen-stimulated improvement in angiogenesis of the tumor by the host animal is a potential mechanism for the estrogen-stimulated growth of the ER- subclone 10A tumors in athymic mice (3,22). Future studies will aim to address this by collaboration to determine blood flow by using labeled microsphere uptake by the ER- tumors in mice, and by morphometry of tumor capillary area, in tumors that develop without and with estrogen. We will determine if angiogenesis as measured by tumor blood supply is altered in both the *SCID*/ER-positive and *SCID*/ER-minus mice.

Analysis of Host (Growth & Apoptotic) Factors Involved in Breast Cancer

Estrogens are believed to be capable of working indirectly by inducing a response in mammary gland mesenchymal cells, which in turn results in a response in neighboring epithelial cells (23). Elucidation of the factors involved in indirect estrogen action is an area of intense study.

Possible areas of research to study estradiol-induced host factors include:

1. Crossing our animals or "traditional" *SCID* mice with "Growth Factor/Apoptotic Knock-out" mice and examining the effects of estrogen on host stimulation of breast tumor growth.
2. Crossing our animals with traditional transgenic mice expressing oncogenes or growth factors (24) known to induce mammary cancer including metastatic disease and then examining the effects of estrogen on growth.
3. Use of growth factor specific monoclonal antibodies *in vivo* to determine if they block host contributions after estrogen stimulation.

4. As in #3, confirm a factor's involvement in breast tumor growth by supplementation with growth factors to determine if they stimulate growth in our ER-, *SCID* background and to also rule out potential growth inhibitory factor mechanisms (25).

5. Analyze breast tumors by differential display PCR (26) both *in vivo* and *in vitro* with varying estrogen treatments to begin the understanding of the interplay of the regulatory gene networks involved. Differential display PCR will identify mRNA sequences that are expressed differently after estrogen stimulation of tumor growth, growing either in ER-negative or ER-positive athymic mice. This powerful technique may make it possible to determine growth response genes in the breast tumor that are being stimulated by estrogen in the host.

Steroid Specificity/ New estrogen responsive proteins or orphan receptors

Various estrogens and antiestrogens may stimulate different patterns of growth and apoptotic factors perhaps through novel protein receptors. Our model system provides a background in which to distinguish the functions of the classic estrogen receptor from those of potentially novel receptors, such as the previously difficult to study "tamoxifen", Type II, and catechol estrogen receptors.

ER Reactivation in *in vitro* ER-negative tumors

The detection of ER protein and mRNA in the breast tumor cell lines we are examining would raise two more questions (at least!).

A. If the all of the human tumor cell lines really contain ER both *in vitro* and *in vivo*, then the question becomes: "Why does the cell respond *in vivo* and not *in vitro*?" or restated:
"Are additional growth factors from the mouse necessary for estradiol to induce a response?". (See above.)

B. If the tumor cell line only contains ER *in vivo* but not *in vitro*, then the question becomes: "What is the mechanism by which ER protein is induced *in vivo* so that cells regain their capacity to respond to estradiol *in vivo* but not *in vitro*?" Recently it has been found that the inactive ER gene expression can be induced *in vitro* by changes in DNA methylation (27,28). It will be extremely interesting to see if a growth or host factor is changing ER gene expression perhaps by an *in vivo* change in ER gene methylation.

Personnel Supported by this Award:

Dennis Lubahn	PI
Wade Welshons	co-PI
Julia Taylor	Post-doctoral Fellow/Technician
Doug Laux	Technician and graduate student
Martin Perry	Technician
Scott Kimsey	Graduate student

Numerous undergrads that helped with animal husbandry

Conclusions:

We have successfully developed assays and analyzed for the recently discovered ER-beta mRNA (17b). These assays will allow us to analyze tumors for *in vivo* activation of this receptor as well as ER-alpha. We have recently discovered evidence for a potential third "ER-gamma" (17c) that responds to catechol estrogens but not estradiol. We will thus need to analyze for differential responses of estradiol *vs.* catechol estrogens.

DNA methylation assays have been developed to analyze the ER gene, as well as other DNA methylations throughout the genome [See paper in Appendix last year (17d) and new paper in Appendix this year (17e)]. This will allow us to look for non-mutational activations of the ER gene IF this methylation site is found in the remaining time of this grant.

Finally, a potentially better and more valuable new mouse model, the SCID/ERαKO mouse, has been developed for the scientific community to allow the study of estrogens and xenoestrogens in breast cancer. This will replace the technically unusable *nude*/ERαKO mouse model proposed in our original study. Studies of estrogens' effects on the immune system have begun.

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Appendices:

1) A Cancer Research paper was attached last year: Huang *et al.* 1997. (Reference 17d).

2) A new Breast Cancer Research and Treatment paper is attached. Laux *et al.* 1999 (Reference 17e).

3) Abstract for 2nd ERA of Hope meeting in October 2000 follows.

Estrogen and Natural Killer Cell Function in ERAKO/SCID/C57BL6J Mice

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Epidemiological data suggests that estrogens play a crucial role in both the promotion and progression of human breast cancer, but the mechanisms by which estrogens act are unknown. We have received support from the U.S. Army Breast Cancer Program to breed an estrogen receptor-alpha (ERα) knockout (ERαKO/SCID/C57BL6J) mouse for the purpose of implanting human breast cancer cell lines in these mice to evaluate the role of host versus tumor ERα in promotion and progression of breast cancer. While we have not been able to grow human breast cancer cells in ERαKO/SCID mice due to technical difficulties, this project led us to studies of the role of ERα and ERβ in Natural Killer (NK) cell function. The ERαKO/SCID animals produced for the above project provided the link to the studies on immune function. We observed an altered immune responsiveness in the ERαKO/SCID mice and because these animals lack functioning T and B cells we reasoned that the innate immune system, including NK cells may be involved in this altered response.

Estrogens are known to promote growth of normal breast tissue as well as various cancer cell lines and obviously this is an area of intense study. Another potential avenue by which estrogens may influence the promotion and progression of breast cancer is by altering immune function and indeed previous studies have demonstrated that estrogen can alter various aspects of the immune system, including thymus development and NK cell function. NK cells are a critical component of the defense against cancer and estrogens have been shown to depress NK cell activity. In order to elucidate the mechanism(s) by which estrogens affect NK cell activity we have examined expression of ERα and ERβ in NK cells. We used flow cytometry to isolate a >90% pure population of NK cells from spleens of WT/SCID mice. We show that by RT-PCR that ERβ mRNA, but not ERα mRNA, is expressed in NK cells. Non-NK cells isolated from spleens are negative for both ERα and ERβ mRNA. Immunocytochemistry confirmed the presence of ERβ protein in isolated NK cells. We also studied NK cell function in ERαKO and wild type (WT) mice. The expression of ERβ in NK cells is very interesting in light of functional analysis of NK cell activity in WT and ERαKO females. NK cells from ERαKO females demonstrate increased killing of YAC-1 (mouse lymphoma) cells in cell-mediated cytotoxicity assays. Further studies are underway to determine whether the increased killing is a result of increased ERβ stimulation due to the high estradiol levels present in ERαKO females or whether the lack of ERα signaling mediates NK cell activity by an indirect mechanism. (The U.S. Army Medical Research and Materiel Command under DAMD17-96-1-6055 supported this work.)

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Report

Hypermethylation of the Wilms' tumor suppressor gene CpG island in human breast carcinomas

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Key words: breast cancer, CpG island, DNA hypermethylation, Wilms' tumor suppressor gene

Summary

CpG island hypermethylation is known to be associated with transcriptional silencing of tumor suppressor genes in neoplasia. We have previously detected aberrantly methylated sites in the first intron of the Wilms' tumor suppressor (*WT1*) gene in breast cancer. In the present study, we extended the investigation to a CpG island located in the promoter and first exon regions of *WT1*. Methylation of this CpG island was found to be extensive in MCF-7 and MDA-MB-231 breast cancer cells, as well as in 25% (five of 20 patients) of primary breast tumors. While levels of the known 3.0-kb *WT1* mRNAs were decreased or not detected in these cell lines, the expression could be partially restored following treatment with a demethylation agent, 5-aza-2′-deoxycytidine. Surprisingly, a novel 2.5-kb *WT1* transcript was expressed at high levels in both untreated and treated MDA-MB-231 cells. This novel transcript was likely a *WT1* variant missing the first exon, and therefore escaped the methylation control present in the normal transcript. Our study implicates the future need to investigate the significance of this aberrant transcript as well as the role of *WT1* CpG island hypermethylation in breast neoplasia.

Introduction

An increase in the cellular capacity to selectively methylate genomic DNA has been observed in breast cancer cells [1]. The event usually occurs at the fifth carbon position of a cytosine located 5′ to a guanine in what is known as a CpG dinucleotide [2, 3]. The dinucleotides are clustered frequently in 1–2-kb regions, known as CpG islands, around the promoters and the first exons of genes [4]. Aberrant DNA methylation in these regions has been shown to provide an alternative pathway, in addition to deletions or mutations, for the silencing of tumor suppressor genes in a variety of cancer types [5–9]. In breast cancer, this epigenetic phenomenon is associated with the inactivation of the estrogen receptor (ER) gene expression in ER-negative cancer cells [1, 10] and with resistance to hormonal therapies in some patients [11].

Our laboratory has been developing strategies to identify novel CpG islands associated with aberrant methylation during the progression of breast neoplasia. Using the methylation-sensitive restriction fingerprinting technique [12], we previously identified a DNA fragment containing the first intron of the Wilms' tumor suppressor (*WT1*) gene, which was frequently hypermethylated in primary breast tumors but unmethylated in the normal breast tissue of the same patients. Since the 5′ end of this gene met the criteria of a CpG island [13], we hypothesized that the *WT1* gene is a candidate for inactivation via methylation silencing in breast cancer.

WT1 was first identified as a tumor suppressor gene by positional cloning on chromosome 11p13 in association with Wilms' tumor, a nephroblastoma common to children [14, 15]. The *WT1* gene is known to express four splice variants, each approximately 3 kb in length [14, 15]. As nuclear transcription factors, the *WT1* proteins modulate expression of a variety of growth factors and their receptors [16]. Functional analysis has demonstrated that the *WT1* gene plays

a crucial role in the mesenchymal-to-epithelial cell transition in the embryonic kidney as well as in other developing tissues of mesothelial origin [17]. Although loss of *WT1* expression was initially associated with the development of Wilms' tumor, additional reports have correlated abnormal *WT1* expression with leukemias, melanomas, mesotheliomas, and ovarian cancers [18–21].

Recently, *WT1* protein was found to be present in normal breast tissues, but was absent or greatly reduced in a subset of primary breast tumors [22]. These findings, coupled with our previous methylation analysis near the 5′ end of the *WT1* gene [12], suggested that methylation silencing is a potential mechanism for *WT1* gene inactivation in breast cancer. In this study, we show that hypermethylation of the *WT1* CpG island was extensive in MCF-7 and MDA-MB-231 breast cancer cells, and in a subset of primary breast tumors. This aberrant event was associated with transcriptional suppression of normal *WT1* transcripts in the cell lines. In addition, we detected a novel transcript that was not subject to methylation silencing and was expressed at a high level in MDA-MB-231 cells.

Materials and methods

Cell culture and tissue samples

The MCF-7 and MDA-MB-231 breast cancer cell lines were obtained from Dr V.C. Jordan (Northwestern University); ZR-75-1 and Hs578t breast cancer cells were obtained from the American Type Culture Collection. MCF-7, MDA-MB-231 and Hs578t cells were routinely maintained in Eagle's minimum essential medium containing 10% fetal bovine serum. ZR-75-1 cells were maintained in RPMI 1640 medium with 10% fetal bovine serum. 5-aza-2′-deoxycytidine (deoxyC) was obtained from Sigma, and freshly prepared in distilled water. Cells were plated at a density of 2×10^4 cells/cm^2 and treated continuously with 0.75 μM deoxyC for two and six days. Control cultures were maintained in the absence of deoxyC. Breast tumor specimens were obtained from patients undergoing mastectomies or biopsies at the Ellis Fischel Cancer Center (Columbia, MO). Adjacent, non-neoplastic breast tissue was obtained from the same patient to serve as a normal control. The patient study was approved by our institutional review board (IRB# 5326).

WT1 probe preparation

A probe, WT1–6 (425 bp), was obtained from *WT1*'s first exon (positions 361–785; GenBank accession no. ×51630) using PCR with the following primers: 5′-TCC GGG TCT GAG CCT CAG CAA A (sense strand); 5′-CCC GTC CAT CCC GCG CAA TCC (antisense strand). The second probe, WT1-7, was an amplified fragment (481 bp) between exons 7 and 10 of the gene using primer sequences previously described [23]: 5′-GGC ATC TGA GAC CAG TGA GAA (sense strand) and 5′-GAG AGT CAG ACT TGA AAG CAG T (antisense strand). PCR products were subcloned into the pCR2.1 cloning vector (Invitrogen) and the inserts were confirmed by DNA sequencing. WT1–6 and WT1–7 were ^{32}P-labeled by the random primer method (Amersham) and used as probes for southern and northern hybridization, respectively.

Southern hybridization

For the analysis of *WT1* CpG island methylation status, 5 μg of genomic DNA from cell lines or patients' specimens were digested to completion with *Mse* I alone or subsequently with methylation-sensitive *Bss*H II, *Sac* II, or *Bst*U I (New England Biolabs). For the structural analysis of the *WT1* CpG island in MDA-MB-231 cells, 5 μg of genomic DNA from this cell line was independently digested with *Pvu* II, *Taq* I, and *Xho* I (New England Biolabs). The digestions were performed as per the supplier's protocols. The restriction products were electrophoresed on a 1.0% agarose gel and transferred to a nylon membrane (Schleicher and Schuell). Membranes were hybridized with the WT1-6 probe. Prehybridization, hybridization, and washing were performed essentially as previously described [24]. Membranes were exposed to Kodak BioMax film in the presence of an intensifying screen for seven days at −70°C. Alternatively, membranes were exposed in a PhosphorImager (Molecular Dynamics) and the results were analyzed using the Image Quant software (Molecular Dynamics).

Northern hybridization

Total RNA was isolated from the breast cancer cell lines using RNeasy total RNA kit™ (Qiagen). Twenty micrograms of RNA were electrophoresed on a 1.4% agarose gel in the presence of 2.2 mM formaldehyde and transferred to a nylon membrane. The membrane

was hybridized with the WT1–7 probe. Hybridization and washing conditions were used as previously described [24]. The membrane was exposed to Kodak BioMax film for eight days at −70°C. Alternatively, the membrane was exposed in a PhosphorImager and the results were analyzed using the Image Quant software. The same membrane was reprobed with a ^{32}P-labeled *β-actin* cDNA probe (1.1 kb) to compare loaded RNA levels. The *β-actin*-probed membrane was similarly examined by PhosphorImager analysis.

Reverse transcription (RT)-PCR

For each sample, 4 μg of total RNA was reverse-transcribed using oligo(dT) primers with the Superscript Preamplification System (Life Technologies). *WT1* primers were designed to amplify an exon 1 fragment (275 bp; sense strand: 5′-TGG GCA GGT AGG GCG CGT TAG GAA; antisense strand: 5′-CGC TCC GGC TTA CGG GTC GTT GG) and a fragment (154 bp) between exons 7 and 8 (sense strand: 5′-AAC GCC CCT TCA TGT GTG C; antisense strand: 5′-GCT GGT CTG AAC GAG AAA ACC TTC). Primer sequences designed for a *β-actin* cDNA fragment (541 bp) were 5′-GTG GGG CGC CCC AGG CAC CA (sense strand) and 5′-GTC CTT AAT GTC ACG CAC GAT TTC (antisense strand). In order to allow for comparison of *WT1* levels between samples, simultaneous PCR amplifications for *WT1* and *β-actin* cDNAs were performed with cDNA (250 ng) in 30 μl volume, containing a pair of *WT1* primers (0.6 μM each) and *β-actin* primers (0.06 μM each), 0.8 units Deep Vent polymerase (New England Biolabs), 200 μM dNTPs, 1 μCi [α−^{32}P] dCTP (3000 Ci/mmole; Amersham) in a buffer provided by the supplier. A touchdown PCR program was employed using the cycling parameters: 96°C for 40 s, 64°C −0.5°C per cycle for 30 s, and 50 s at 72°C for 22 cycles. Under this low cycling condition, amplification was expected to be in the linear range of the assay. PCR products were size-fractionated on a 4.5% nondenaturing polyacrylamide gel. After electrophoresis, wet gels were wrapped with plastic film and exposed to Kodak BioMax film at −70°C. Autoradiography for *WT1* and *β-actin* cDNAs was carried out for 15 h. The levels of *WT1* cDNAs were normalized with the level of *β-actin* product band in the respective samples. RT-PCR was independently performed at least two times with different RNA sources.

Results

Methylation analysis of the WT1 CpG island in breast cancer cell lines

We examined the methylation status of the *WT1* promoter and first exon regions in MCF-7 and MDA-MB-231 cells by southern hybridization. As shown in Figure 1a, this region displays characteristics of a CpG island, being highly GC-rich with a G+C content of 70% and having a CpG/GpC ratio of 74%. The region includes many recognition sites for methylation-sensitive endonucleases, including two *Bss*H II, one *Sac* II, and 12 *Bst*U I sites. These sites are located within a 1.6-kb fragment flanked by two recognition sequences for *Mse* I. *Mse* I cuts genomic DNA into small fragments (once per ~140 bp), leaving CpG islands generally intact [25]. As expected, digestion of genomic DNA with *Mse* I alone produced a 1.6-kb fragment (Figure 1b, lanes 1, 5, and 9). An additional fragment with slightly less molecular weight than the 1.6-kb fragment was observed in the control sample (lane 1). This may represent a polymorphism, as this smaller band was also detected in breast tissue from patients 67 and 127 (Figure 2). Double digestion of the control DNA with *Mse* I and each of the three methylation-sensitive endonucleases, *Bss*H II (lane 2), *Sac* II (lane 3), and *Bst*U I (lane 4) yielded the expected 1.4−, 0.9−, and <0.2-kb fragments, respectively, demonstrating that these sites were not methylated in the control sample. In contrast, the 1.6-kb *Mse* I-restricted fragment appeared to be protected from the methylation-sensitive restrictions (lanes 6–8) in the MCF-7 samples, suggesting that each of these sites was completely methylated within the *WT1* CpG island of this cell line. A similar southern hybridization result was obtained in the MDA-MB-231 samples (lanes 10–12), except that retention of additional minor fragments with smaller molecular weights in the *Mse* I/*Sac* II and *Mse* I/*Bst*U I digests was observed. This could be attributed to the cellular heterogeneity in the MDA-MIB-231 cell line, showing the presence of a few unmethylated CpG sites in some cells.

Methylation analysis of the WT1 CpG island in primary breast tumors

Methylation analysis by southern hybridization was undertaken on breast tumor samples from 20 patients with infiltrating ductal carcinomas. One methylation-sensitive enzyme, *Bst*U I, was used in the analysis because of the limited amount of available patient

A

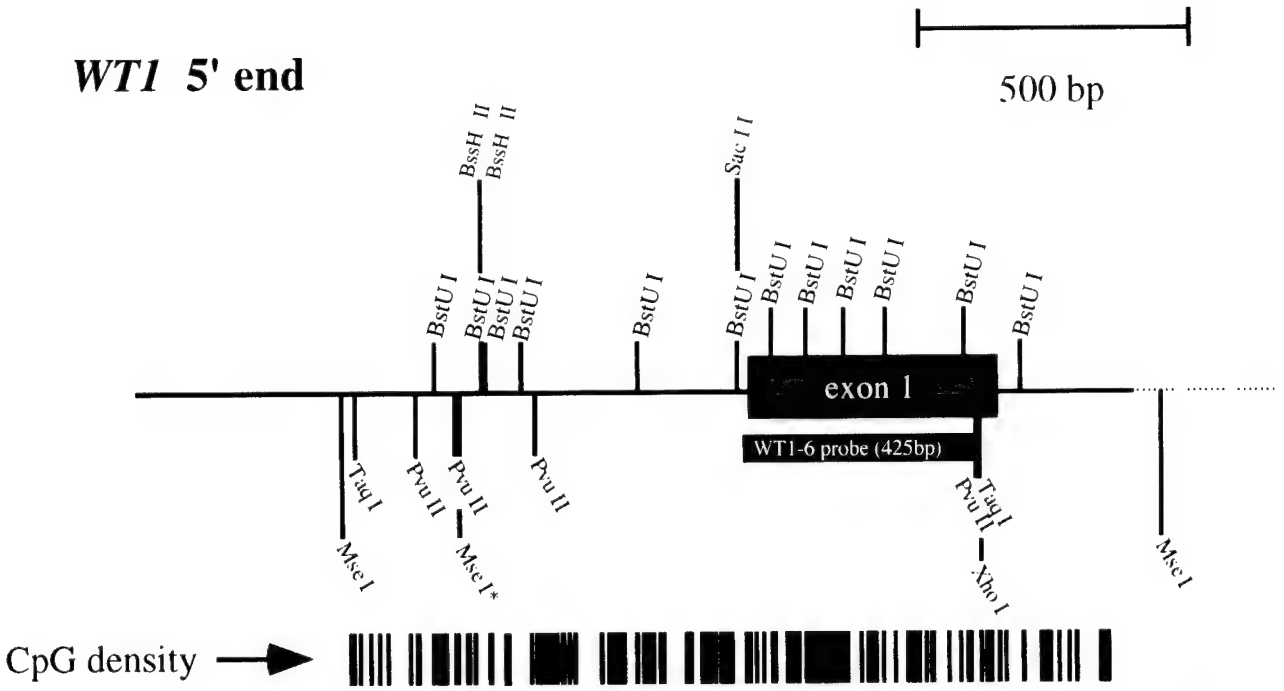

Figure 1a. Schematic representation of the *WT1* CpG island in the promoter and first exon regions. The relative positions of the methylation-sensitive restriction sites for *Bst*U I, *Bss*H II, and *Sac* II as well as other methylation-insensitive restriction sites (*Mse* I, *Pvu* II, *Taq* I, and *Xho* I) are indicated. The relative density of CpG dinucleotides is also indicated. A fragment spanning the first exon, WT1–6, was used as a probe in southern hybridization. The *Mse* I fragment is approximately 1.6 kb in length. The dotted line indicates DNA sequence not reported in the literature. **Mse* I denotes a polymorphic site observed in some patients.

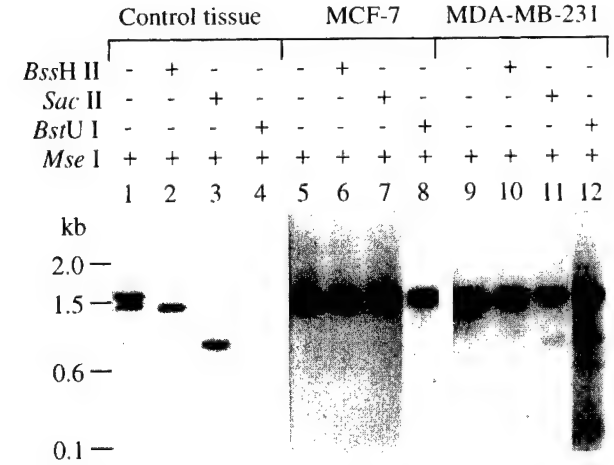

Figure 1b. Methylation analysis of breast cancer cell lines MCF-7 and MDA-MB-231 as well as normal control tissue by southern hybridization. Genomic DNA (5 μg) was treated (+) or untreated (−) with restriction endonucleases as indicated and subjected to southern hybridization analysis using WT1–6 as the probe. Molecular weight markers (100-bp ladder; Life Technologies) are shown at left.

DNA. Restriction of genomic DNA with this endonuclease also enabled the examination of a greater number of possible methylated cytosines within the *WT1* CpG island (see Figure1a). Genomic DNA samples from breast tumors were digested with both *Mse* I and *Bst*U I, and hybridized with the WT1–6 probe. DNA samples from available matching normal tissues were similarly analyzed. Figure 2 shows representative results for seven of these patients. The double-digested tumor DNA from patients 29, 69, 119, and 127 hybridized to the 1.6-kb fragment, indicating complete methylation of all *Bst*U I sites within the *WT1* CpG island as described earlier. Methylation of the island was not observed in the tumor DNA of patients 133 and 135. Additional smaller-sized fragments were detected in patient 29 due to the presence of unmethylated *Bst*U I sites in some of the patient's tumor cells. The DNA from patient 89 hybridized to a smaller fragment (~1.0 kb), indicating partial methylation of the *WT1* CpG island in this tumor. Methylation of the *WT1* CpG island was not detected in the matching normal samples from these patients, although normal tissue

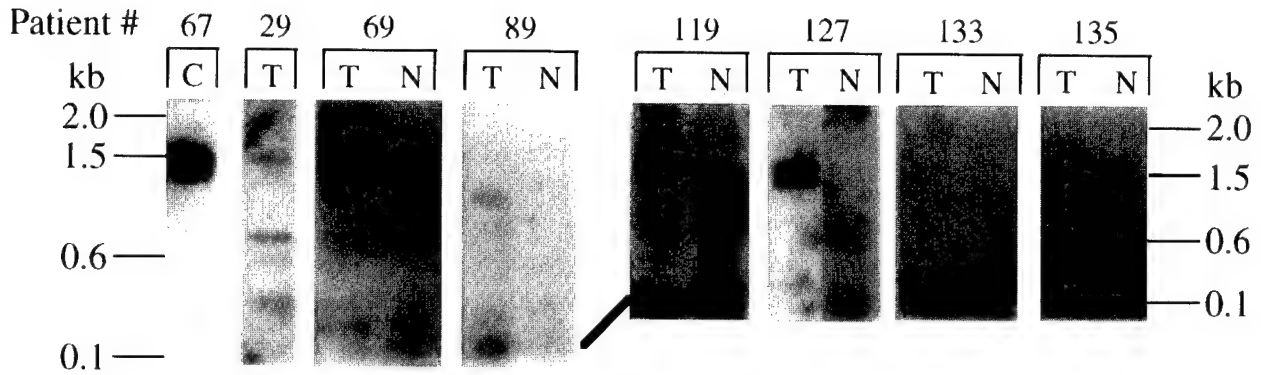

Figure 2. Methylation analysis of the *WT1* gene in primary breast tumors (T) and the matching normal breast tissues (N) from patients by southern hybridization. No normal control was available for patient 29. Genomic DNA (5 μg) was treated consecutively with *Mse* I and *Bst*U I, then subjected to southern hybridization analysis using *WT1*-6 as the probe. C: Control DNA digested with *Mse* I only. Representative molecular weight markers (from the 100-bp ladder; Life Technologies) are shown flanking both sides of the figure.

Table 1. Clinical information and methylation studies of female patients with infiltrating ductal carcinoma of the breast

Patient no.	Age at diagnosis (yr)	Clinical staging[a]	Methylation status of *WT1* in tumor[b]
29	77	I	+
69	37	IIa	+
73	57	IIa	–
81	72	IIb	–
89	83	IV	+
101	91	I	–
103	67	IIa	–
107	47	IIa	–
109	66	IIa	–
111	70	IIIa	–
113	81	IIa	–
117	46	IIIa	–
119	67	IIIa	+
121	52	IIIa	–
123	38	IIb	–
127	35	IIIb	+
129	76	IIIb	–
131	80	ND	–
133	44	IIb	–
135	46	IIa	–

[a]Clinical staging was according to the criteria of the American Joint Committee on Cancer [33].
[b]+, hypermethylation in tumor; –, lack of methylation in tumor (see additional description in the text).

from patient 29 was unavailable for study. The results of this methylation analysis and the patients' clinical information are summarized in Table 1.

Methylation silencing of the WT1 gene in breast cancer cell lines

We next investigated whether the *WT1* CpG hypermethylation correlated with down-regulation of the *WT1* gene in MCF-7 and MDA-MB-231 cells. Cells were treated with a demethylating agent, deoxyC, and patterns of their *WT1* gene expression were determined by northern hybridization. Initial analysis using probe WT1–6 produced non-specific hybridization patterns, and was attributed to this probe's highly GC-rich nature. A second probe, WT1–7, specific to *WT1* exons 7–10 was generated for northern hybridization. WT1–7 detected a band migrating at 3.0-kb corresponding to the size of the previously described *WT1* mRNAs [26] in the control cell lines ZR-75-1 (lane 1) and Hs578t (lane 3). These cell lines exhibited no hypermethylation of the *WT1* CpG island region by prior southern analysis (data not shown), and a 6-day treatment of both cell lines with deoxyC resulted in no change in *WT1* expression. The 3.0-kb transcripts were faintly detectable in the MCF-7 untreated cells (lane 5), and this expression level slightly increased following treatment with deoxyC for six days (lane 7). The partial restoration of *WT1* expression suggests that either the deoxyC dosage was inadequate to effectively demethylate the *WT1* promoter and thereby induce full expression, or that other factors in addition to methylation suppress *WT1* transcription in the MCF-7 cell line. Both untreated and 2-day treated MDA-MB-231 cells expressed no detectable 3.0-kb transcripts (lanes 8 and 9). However, treatment of MDA-MB-231 cells for six days resulted in a greater increase in expression of 3.0-kb transcripts than in MCF-7 cells (lane 10). Surprisingly, a *WT1* mRNA variant with a smal-

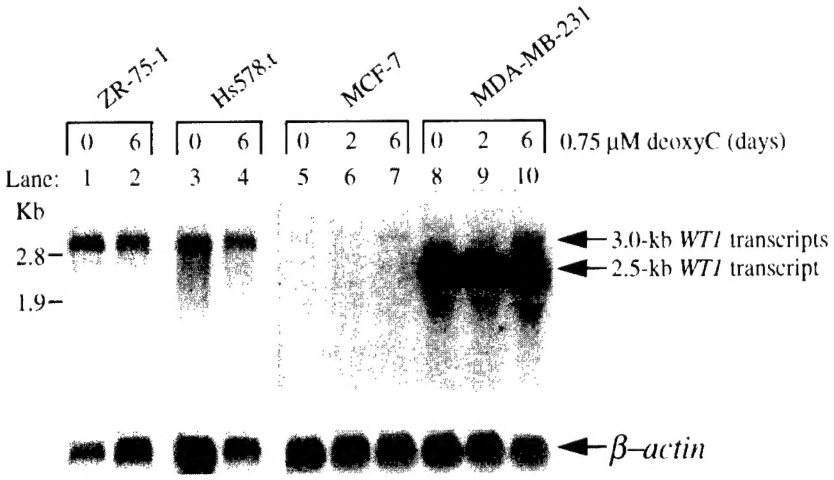

Figure 3. Northern hybridization analysis of *WT1* gene expression in breast cancer cells. ZR-75-1, Hs578t, MCF-7, and MDA-MB-231 cells were either untreated or treated with 0.75 μM 5-aza-2′-deoxycytidine (deoxyC) for two or six days as indicated. Total RNA (20 μg) from these cells was subjected to northern hybridization analysis with a cDNA probe, WT1–7, containing *WT1* exons 7–10 (481 bp). The filter was reprobed with *β-actin* cDNA (1.1 kb). Representative molecular weight markers (from the RNA MW I ladder, Boehringer Mannheim) are shown at left.

ler molecular weight was identified at a high level in MDA-MB-231 cells, and its expression was not altered by deoxyC treatment. Based on a shorter autoradiographic exposure, the message was estimated to be 2.5-kb. All samples were assayed for the *β-actin* mRNA level to enable a relative comparison of loaded RNA amounts (Figure 3, lower panel).

Characterization of the 2.5-kb transcript in MDA-MB-231 cells

RT-PCR analysis aided in identifying the possible composition of the novel 2.5-kb transcript in MDA-MB-231 cells (Figure 4). Amplification product derived from *WT1* exon 1 was not detected in the untreated cells, but was observed in the cells treated with deoxyC (upper panel). The amplified product spanning exons 7 and 8, however, was present in the untreated cells and at a greater level in the treated cells (lower panel). Both cDNA products of exon 1 and exons 7 and 8 were equally amplified in a control sample. The fact that RT-PCR failed to amplify an exon 1 product but amplified cDNA across exons 7 and 8 in the untreated MDA-MB-231 cells indicated that the 2.5-kb transcript described above may not contain the *WT1* first exon. As further evidence of this fact, the first exon has been reported as 454 bp [26], and our Northern analysis demonstrated that this transcript was ~500 bp shorter than the normal 3.0-kb transcripts. The amplified product detected in the treated cells using the *WT1* exon 1 primers represented the 3.0-kb transcripts restored by the demethylation treatment. This observation is consistent with the result that a greater level of cDNA products from exons 7 and 8 was present in the treated cells than in the untreated cells due to the re-expression of the 3.0-kb transcripts.

To determine whether a gene rearrangement or chromosomal translocation involving the *WT1* 5′ end was responsible for expression of the 2.5-kb transcript, restriction mapping analysis of the *WT1* promoter and first exon regions was performed (Figure 5). The known restriction sites of the three endonucleases used in the Southern hybridization (*pvu* II, *Taq* I, and *Xho* I) are indicated in Figure 1A. The results show that the restriction patterns of both MDA-MB-231 cells and normal breast tissue were the same, suggesting that no major structural alterations of the promoter and first exon regions have occurred in MDA-MB-231 cells.

Discussion

The present study demonstrated that the *WT1* CpG island was hypermethylated in MCF-7 and MDA-MB-231 breast cancer cells. Treatment of these cells with a demethylating agent, deoxyC, partially restored *WT1* expression, providing an association between CpG island hypermethylation and *WT1* gene silencing in the cell lines. We have further shown *in vivo* that the *WT1* CpG island was hypermethylated in 25% (five out of 20 patients) of the examined breast tumors. Thus,

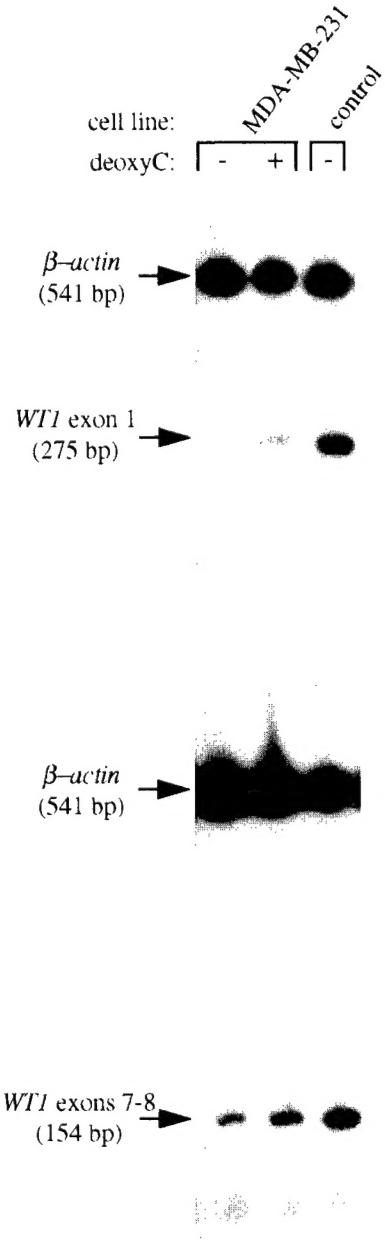

Figure 4. RT-PCR analysis of *WT1* gene expression in MDA-MB-231 cells. cDNA was reverse-transcribed from 4 μg total RNA using oligo(dT) primers and the Superscript Preamplification System (Life Technologies). Diplex PCR amplification using primer sets either for *WT1* exon 1 and *β-actin* (upper panel) or for *WT1* exons 7 and 8 and *β-actin* (lower panel) was performed as described in the text.

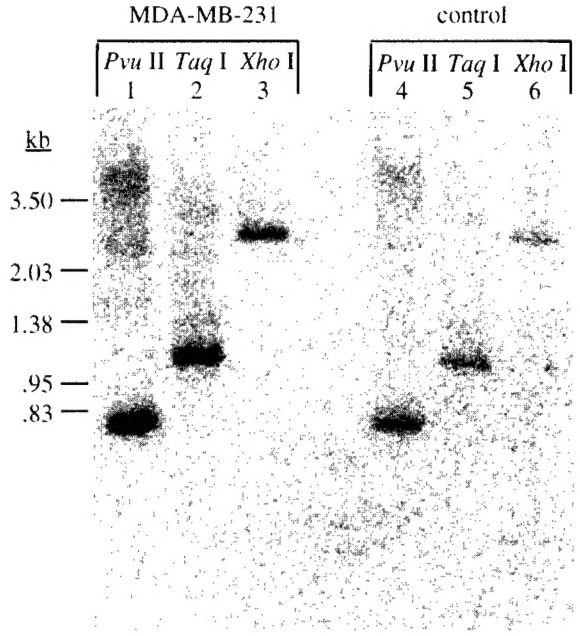

Figure 5. Physical mapping of the *WT1* 5′ region including the promoter and first exon in MDA-MB-231 cells by southern hybridization. Genomic DNA (5 μg) from both MDA-MB-231 cells and a normal breast tissue control sample was separately digested with *Pvu* II(P), *Taq* I (T), and *Xho* I (X), which have known recognition sites within the region of the *WT1* promoter and first exon (see Figure 1a). Hybridization was performed using the WT1–6 probe. Molecular weight markers (MW III, Boehringer Mannheim) are shown at left.

this study adds the *WT1* gene to a growing list of tumor suppressor genes associated with transcriptional down-regulation by DNA methylation [5–9].

Our data suggest that the novel 2.5-kb transcript identified in MDA-MB-231 cells is a *WT1* mRNA variant and that its expression is insensitive to the methylation state of the *WT1* gene (Figures 3 and 4). This shorter transcript may not contain exon 1, and expression of this novel transcript appears not to be influenced by the hypermethylation state of the promoter and first exon regions in the *WT1* gene. Future investigations will focus on cloning and verifying the cDNA sequence derived from this transcript, as well as on characterizing any altered functions of the resulting truncated protein in breast cancer.

Recent evidence indicates a developmental role for *WT 1* in the normal breast. Using immunohistochemical methods, Silberstein et al. [22] reported that *WT1* protein was detectable in mature normal mammary ductal and lobular epithelia, with the protein most abundant in less-differentiated putative stem cells, suggesting that *WT1* plays a differential regulatory role in the development of these mammary structures. Functionally, WTl can negatively regulate the expression of the insulin-like growth factor I receptor, insulin-like growth factor II [27–28] and the transforming growth factor β genes [29], all of

which are important in normal breast development. *WT1* inactivation in breast cells could result in an overexpression of these genes, thereby promoting tumorigenic processes. Indeed, Silberstein et al. [22] further observed absent or greatly reduced levels of WTl protein in 68% of a set of 21 primary breast tumors. While the contributions of *WT1* gene deletions, mutations, and antisense suppression [30] to the loss of *WT1* function remain undetermined, our results suggest that hypermethylation of the *WT1* CpG island can be an effective mechanism in attenuating *WT1* expression in breast cancer.

Although this study has focused on breast cancer, suppression of *WT1* expression through hypermethylation may play a role in the development of Wilms' tumor and other forms of human neoplasia. Pulsed-field gel electrophoresis has been used to identify aberrantly methylated sequences within a 300-kb chromosomal region containing the *WT1* gene in Wilms' tumors [31]. More recently, hypermethylation of the *WT1* gene promoter was found in primary colonic adenomas and carcinomas [32]. As *WT1* is expressed in a variety of normal tissues, hypermethylation may provide a common mechanism for modulating *WT1* in a number of different cancer types.

In summary, we have shown that hypermethylation of the *WT1* CpG island was associated with silencing of full length (or normal) *WT1* mRNA expression in MCF-7 and MDA-MB-231 breast cancer cells. Future studies will focus on correlating *WT1* CpG island methylation with possibly altered *WT1* gene expression in primary breast tumors. Moreover, we will study the relevance of the novel 2.5-kb *WT1* transcript to breast cancer progression.

Acknowledgements

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